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Use of In Situ-Generated Dimethyldioxirane for Inactivation of Biological Agents

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Dimethyldioxirane (DMDO), generated in situ by adding acetone to an aqueous solution containing potassium peroxymonosulfate (Oxone) at neutral pH, was investigated for inactivation of biological warfare agent simulants. The DMDO solution inactivated bacterial spores, fungal spores, vegetative bacterial cells, viruses, and protein by 7 orders of magnitude in less than 10 min. The kill rates of DMDO were more pronounced when compared to kill rates of buffered Oxone alone. Conditions for the use of DMDO as a biological decontaminant were optimized by evaluating the effects of age and temperature on open systems. DMDO effectiveness was compared to that of current decontaminant solutions such as DS2 (used by the U.S. military), bleach, and hydrogen peroxide and was shown to be superior in achieving a 7-log kill of *Bacillus atrophaeus*, a *Bacillus anthracis* spore simulant. The results demonstrate the potential for DMDO to fill the need for a noncorrosive, nontoxic, and environmentally safe decontaminant.

Introduction

Standard decontamination methods for the armed forces require harsh chemical formulations based on sodium hypochlorite, a strong alkali, and organic compounds that are highly corrosive and toxic (*1*). Concerns about potential adverse effects on the health of exposed individuals, on the environment, and on decontaminated equipment have driven the search for alternative decontaminants for both military and civilian applications. Efforts to develop equally effective, less corrosive, and less toxic decontaminants have focused on strong oxidative aqueous treatments using titanium catalyzed photolysis (*2*), organic and inorganic peracids (*3*–*6*), and activated hydrogen peroxide (*7*, *8*). Each of these decontaminants has specific limitations. Therefore, the search continues for the identification of a decontaminating agent that would be highly effective against all classes of potential chemical and biological weapons while being nontoxic, noncorrosive, and environmentally benign.

Dioxiranes constitute a new class of powerful oxidants. They were first prepared in 1974 (*9*) by reacting peroxymonosulfate with select ketones under controlled pH conditions (Figure 1). Dioxiranes, mainly dimethyldioxirane (DMDO), have since been used extensively to carry out a variety of synthetically useful oxidations under mild conditions (*10*–*12*).

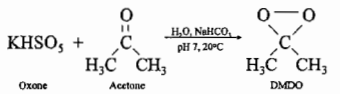

FIGURE 1. Reaction of Oxone (active component, $KHSO_5$) and acetone (CH_3COCH_3) to form aqueous DMDO ($CH_3C(OO)CH_3$) at room temperature and at neutral pH in the presence of sodium bicarbonate ($NaHCO_3$).

DMDO is prepared in an in situ aqueous system that has dynamic properties. Peroxymonosulfate will autodecompose in solution to evolve oxygen and precipitate sulfate and undergo catalytic decomposition in the presence of acetone to generate DMDO. Experiments using ^{18}O-labeled reagents have shown that acetone is recycled in the system after DMDO oxidizes a target substrate (*13*). Methyl acetate has recently been identified as a product in the in situ DMDO matrix (*14*), and its formation has been suggested to proceed through a radical intermediate generated from acetone (*15*). Regardless, available acetone in the system will continue to react as long as active oxygen is available from peroxymonosulfate.

The optimal pH for dioxirane preparation is in the neutral range, and the byproducts, potassium sulfate and sodium bicarbonate, are environmentally benign. No toxic byproducts from the DMDO formulation have been reported to date, and acetone is no longer listed by the U. S. Environmental Protection Agency (EPA) as a hazardous air pollutant (*16*). These attributes provide the incentive to pursue in situ-generated DMDO as a decontaminant formulation that can potentially minimize impact on equipment, personnel, and the environment.

Surrogates are commonly used in experiments to simulate biological warfare agents. Bacterial spores are deemed ideal substrates for efficacy testing because they are the form of life most resistant to adverse physical and chemical environments (*17*). Spores of *Bacillus atrophaeus* (formerly known as *B. subtilis globigii*) (*18*) are widely used as surrogates for *B. anthracis* spores (*19*, *20*). The spore coat of *B. atrophaeus* has a structure similar to that of *B. anthracis*, consisting of a complex multilayer of glycoproteins. Another spore-forming organism similar to *B. anthracis* is *B. thuringiensis*. *Escherichia coli* and *Pseudomonas aeruginosa* are used to simulate the Gram-negative bacteria *Yersinia pestis* and *Francisella tularensis*, which are known to have been stockpiled for weaponization (*21*, *22*). *Aspergillus niger* spores are used to simulate pathogenic fungal spores. Although fungi are not known to have been weaponized, the potential threat to agriculture and food production warrants consideration of this class of microorganisms. MS2 bacteriophage is used to simulate infectious viruses. This small (25 nm) virus (*21*) is more resistant to chemical disinfection than medium sized human-infecting viruses (*23*) and is widely accepted by the decontamination community as a suitable surrogate for pathogenic viruses. Bovine serum albumin (BSA) has been used as a model for bacterial polypeptide toxins such as ricin and botulinum toxin and other bacterial toxins (*24*).

The biocidal properties of dioxiranes have been reported to be excellent in acidic buffer at pH 4, but limited biocidal action was observed at neutral pH in a phosphate buffer (*25*). Therefore, the efficacy of in situ-generated DMDO for inactivation of targeted microorganisms was investigated at

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a neutral pH using sodium bicarbonate as the buffer and compared to that of conventional decontaminants. The main parameters impacting its use in field operations were investigated and discussed.

Materials and Methods

Reagents. All reagents were obtained in the U.S. unless otherwise stated. Oxone (triple salt [$2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$], 48.5% as potassium peroxymonosulfate), sodium bicarbonate (>99.7%), ammonium sulfate (99.8%), ferrous sulfate (98%), 2-mercaptoethanol (>98%), and monobasic potassium phosphate (99%) were purchased from Sigma-Aldrich, Milwaukee, WI. Ultra-Resi analyzed acetone was purchased from J. T. Baker, Phillipsburg, NJ. Magnesium chloride (99%), sodium thiosulfate (>98%), yeast extract (100%), and hydrogen peroxide (35%) were purchased from Fisher Scientific, Fairlawn, NJ. Bovine serum albumin (66 kDa), SDS (70%), polyacrylamide SDS (>90%), Tris buffer (>99%), and glucose 6-phosphate dehydrogenase (254 units/mg) were purchased from F. Hoffmann-La Roche Ltd., Switzerland. A molecular weight marker was purchased from Promega Biosciences, Inc., San Luis Obispo, CA. Daybright bleach (5.5%) was manufactured by KIK International, Houston, TX. DS2 (a mixture of diethylenetriamine [70%], diethylene glycol monomethyl ether [28%], and sodium hydroxide [2%]) was purchased from Poly Research Corp., Holtsville, NY. The source of 18 MΩ-deionized water (DI) was a Milli-Q system (Millipore Corp., Molsheim, France).

Microorganisms. The basal salts medium (BSM) used for all cell, fungi, spore, and bacteriophage experiments contained the following constituents: KH_2PO_4 (50 mM), NH_4SO_4 (15 mM), $MgCl_2$ (1 mM), and $FeSO_4$ (0.02 mM). The microorganisms, *B. atrophaeus* (ATCC 9372), *B. thuringiensis* (ATCC 35646), *E. coli* (ATCC 15597), *A. niger* (ATCC 1004), and MS2 bacteriophage (ATCC 15597-B1), were purchased from ATCC, Manassas, VA. The PAO1 strain of *P. aeruginosa* was obtained from the Pseudomonas Genetic Center, East Carolina University School of Medicine, Greenville, NC.

Preparation of Microorganism Cultures. Spores of *B. atrophaeus* and *B. thuringiensis* were prepared separately by inoculating each organism into a small batch of nutrient broth yeast extract (NBY) medium (8 g/L NB, 3 g/L YE) and growing the culture overnight at 35 °C while shaking at 175 rpm. The culture was used to inoculate 10 L NBY, which was incubated in a 13 L New Brunswick fermentor at 37 °C with an air flow of 10 L/min, until more than 95% of the vegetative cells had been converted into spores (as measured by standard spore stain and Gram stain procedures). Spore preparations were heated to 80 °C for 10 min to eliminate vegetative cells and then suspended in water and stored at 4 °C. The viable spore count was determined by standard dilution and plating methods on nutrient agar plates (*26*). Spore counts of *B. atrophaeus* initial spore preparations were 10^8 to 10^9 colony-forming units (CFU) per milliliter. Spore counts of *B. thuringiensis* initial spore preparations were 10^6 to 10^7 CFU per milliliter.

Spore preparations of *A. niger* were prepared by inoculating 40–50 potato dextrose agar (PDA) plates with a lawn of *A. niger* culture. Plates were allowed to incubate at room temperature for 7–10 days. Conidial spores were then harvested by adding 10 mL of sterile saline solution (5 g/L NaCl, 0.5 g/L Triton X-100) to each plate, scraping the surface of the plate to liberate the spores, and decanting liquid through sterile cheesecloth into a separate container. Spore preps were stored at 4 °C. Spore counts of *A. niger* initial spore preparations were 10^7 CFU per milliliter.

Cultures of *E. coli* were prepared by inoculating sterile NBY and incubating overnight at 37 °C while shaking at 175 rpm. All cultures used were no older than 24 h. Cultures of *P. aeruginosa* were prepared in the same manner. Cell counts of *P. aeruginosa* and *E. coli* initial cultures were 10^8 to 10^9 CFU per milliliter.

MS2 bacteriophage cultures were prepared by adding a 50 mL of overnight culture of *E. coli* ATCC 15597 in specialized MS2 media (10.0 g/L tryptone, 10.0 g/L NaCl, 5.0 g/L yeast extract) to 500 mL of sterile specialized MS2 media to which 2 mL of 1 M $CaCl_2$ had been added. This culture was incubated for 3 h at 35 °C while shaking at 175 rpm, after which 1.5 mL of MS2 phage culture was added. The culture was incubated overnight at 35 °C while shaking at 175 rpm. Lysozyme was added (0.5 mL/L), and the culture was incubated for 30 min at 35 °C while shaking at 175 rpm. EDTA was then added (0.5 mL/L of a 0.5 M solution), and the culture was incubated again for 30 min at 35 °C while shaking at 175 rpm. The culture was centrifuged for 60 min at 800 rpm, and the supernatant was stored at 4 °C. Titer of the bacteriophage prep was determined by serial dilution and the soft agar overlay method (*26, 27*). Titer of initial phage preparation of MS2 bacteriophage was 10^9 to 10^{10} plaque-forming units per milliliter.

Preparation of Oxidant Solutions. The buffered Oxone solutions were prepared by adding solid Oxone (10% w/v) to a sodium bicarbonate solution (4.2% w/v) in deionized water at 22 °C. Because of vigorous off-gassing during the preparation, the solid Oxone reagent was added slowly to the bicarbonate solution and allowed to mix for 10 min before use. The DMDO solutions were made by adding acetone (10% v/v) to the buffered Oxone solutions. All oxidant solutions had a final pH of 7.1 ± 0.1. All oxidant solutions used were prepared as described previously, unless noted otherwise, and were applied to the targeted organism less than 10 min after preparation. DS2, bleach, and hydrogen peroxide were used as received.

Inactivation of Targeted Microorganisms. For all experiments, 8 mL of the oxidant solution (DS2, bleach, hydrogen peroxide, Oxone, or DMDO) was added to either 1 mL of acetone (for DMDO) or 1 mL of deionized water (all other oxidants), followed by a 1 mL addition of the initial microorganism preparation. All reactions were terminated after exposure by adding 1 mL of reactant solution into 1 mL of 1 N sodium thiosulfate. Preliminary experiments show that adding equal volumes of the reactant solution and 1 N sodium thiosulfate results in complete neutralization of the oxidant and that the resulting solution has no further biocidal action on the targeted microorganisms. After quenching the reactions with sodium thiosulfate, the residual count of each targeted microorganism was determined by serial dilution and plating. For MS2 bacteriophage, viability was evaluated by the soft agar overlay method.

Inactivation of Targeted Protein. Preliminary experiments, performed at 22 °C, revealed that the BSA was destroyed within seconds using oxidant solutions comparable to those used against the microorganisms. In an effort to slow the reaction and detect resulting products, the oxidant solution was reduced in strength to 2% (w/v) Oxone and 2% (v/v) acetone. A solution of 40 μg of BSA in 50 μL of deionized water was treated with 50 μL of oxidant solution for 5 min and then quenched with an equal volume of 1 N sodium thiosulfate. Dialysis was performed at 4 °C to separate the products from the oxidants. The concentration was adjusted to 0.6 μg/μL. Twenty micrograms of protein material was added to 45 μL of a solution of 1% (v/v) sodium dodecyl sulfate (SDS) solution and 1% (v/v) 2-mercaptoethanol. This mixture was boiled and loaded onto a 7.5% (w/v) polyacrylamine SDS gel. Electrophoresis was carried out in a Tris-glycine buffer (pH 8.8) at 15 mA for 4 h and then at 4 mA for 16 h. The gels were stained with Coomassie blue, and protein degradation was visually assessed by comparing the protein bands to the control bands, which consisted of buffer alone and 2% (v/v) acetone in buffer.

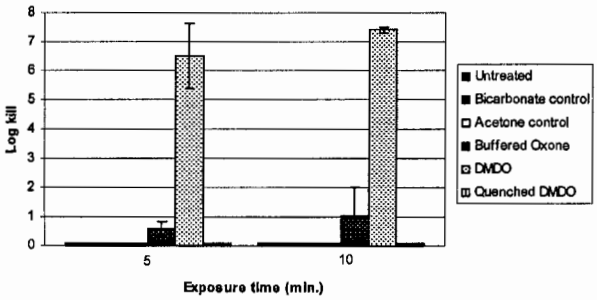

FIGURE 2. Inactivation of *B. atrophaeus* spores over time with 10% buffered Oxone and DMDO solutions, against controls. Error bars indicate standard deviation of three replicates.

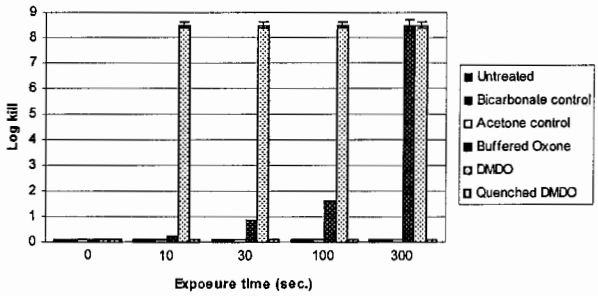

FIGURE 3. Inactivation of *E. coli* cells over time with 10% buffered Oxone and DMDO solutions, against controls. Error bars indicate standard deviation of three replicates.

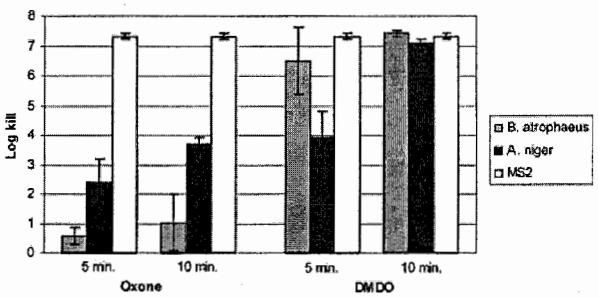

FIGURE 4. Comparison of inactivation of *B. atrophaeus* spores, *A. niger* spores, and MS2 bacteriophage over time with 10% buffered Oxone and DMDO solutions. Error bars indicate standard deviation of three replicates.

Inactivation of Spores on Tire Surfaces. A Goodyear two-ply nylon cord tire was cut into 5 cm^2 coupons. The tire coupons were sterilized in 70% ethanol and allowed to dry overnight. Liquid cultures of *B. atrophaeus* and *B. thuringiensis* spore preparations (30 mL each) were added to separate sterile glass Petri dishes. The tire squares were placed tread-side down in a dish containing spores. After 5 min, the tire coupons were removed from the dish with sterile forceps, and the excess spore solution was gently shaken off. Each tire coupon was transferred to a second glass Petri dish containing either bicarbonate buffer, acetone, 10% (w/v) buffered Oxone solution, or DMDO solution. The tire coupons were allowed to soak in the second solution for 10 min. The dishes were slightly agitated once during the exposure time. After quenching the oxidant, the cell count of the resulting liquid was recorded to quantify the effectiveness of the decontamination.

Results and Discussion

Inactivation Rates of Bacterial Spores. As described in the Materials and Methods, DMDO is commonly produced in situ by adding acetone to an aqueous solution of Oxone and sodium bicarbonate. The sodium bicarbonate buffers the resulting solution to pH 7.1 ± 0.1. This simple buffer is ideal for practical field applications because it is nonhazardous and easy to prepare. Therefore, the first investigation focused on the action of this simple buffered system on the most recalcitrant microbiological life form, bacterial spores. For this study, the targeted inactivation rate was 7-log kill of all microorganisms in 15 min.

When spores of *B. atrophaeus* were exposed to a solution of DMDO, an inactivation rate exceeding 10^7 spores/mL (7-log kill) was achieved after 10 min (Figure 2). A significantly reduced killing rate was observed when Oxone was present but acetone was omitted from the preparation, and little to no kill was observed when Oxone was absent from the solution. These observations suggest that the increase in inactivation rate is due to the action of DMDO itself and not the action of any other reactant used to produce DMDO in situ. This conclusion is also supported by the absence of biocidal action observed when the DMDO solution was treated with a reductive agent, such as sodium thiosulfate, before application to the spores.

The 7-log kill of *B. atrophaeus* by DMDO in 10 min exceeded that of an alternative hydrogen peroxide-based decontaminating solution that required 60 min for an equivalent kill (*22*). Other liquid sporicidal agents, such as peracetic acid, can require up to 30 min to inactivate 99.9% (3 logs) of a *B. anthracis* simulant (*28*) and may yield toxic byproducts.

Inactivation Rates of Vegetative Bacterial Cells. DMDO was extremely effective against vegetative cells, inactivating *E. coli* (Figure 3) and *P. aeruginosa* (results not shown) cells in 10 s or less (8-log kill). Again, it was observed that although Oxone alone at neutral pH was effective in killing vegetative bacteria, DMDO was equally effective and killed at a faster rate.

Microscopic examination of Gram-stained vegetative cells of *P. aeruginosa* following the DMDO treatment did not show any observable change in the overall structure, size, and shape of the cells. However, when stained, the pink color typical of Gram-negative cells was very faint. The DMDO-treated cell preparations were also not viscous, as would be expected if lysis had occurred and DNA and protein molecules had been released into the medium. These observations suggest that DMDO does not disrupt the cell wall and that another oxidative mechanism causes rapid cell death. Treatment of cells with sodium thiosulfate alone, without Oxone or DMDO present, yielded a normal Gram stain.

Inactivation Rates of Fungal Spores and Viruses. The *A. niger* cultures examined consisted of >90% spores. The results for DMDO action on fungal spores were similar to the results observed for bacterial spores. A 7-log kill was achieved with DMDO after 10 min (Figure 4). These observations suggest that the killing mechanism is accelerated by DMDO.

The MS2 bacteriophage was completely inactivated after 10 s exposure to both the buffered Oxone solution and DMDO solution. Since small viruses are typically more resistant to chemical disinfection than medium-sized viruses such as the smallpox virus (*23*); the inactivation rates observed for the tested simulant viruses bode very well for the effectiveness of DMDO as a decontaminant for pathogenic viruses.

Inactivation Rates of Protein-Based Toxins. BSA protein was quickly degraded upon exposure to DMDO at 22 °C. Gel electrophoresis of the treated protein after 1 min exposure (Figure 5) showed no protein remaining in the lane containing DMDO-treated BSA, while protein was clearly visible in the lanes containing BSA treated with bicarbonate buffer or with acetone. In the lane containing Oxone-treated BSA, a faint trace of protein at a slightly increased molecular weight was visible. The small increase in molecular weight suggests a pathway of degradation that might include cross-links

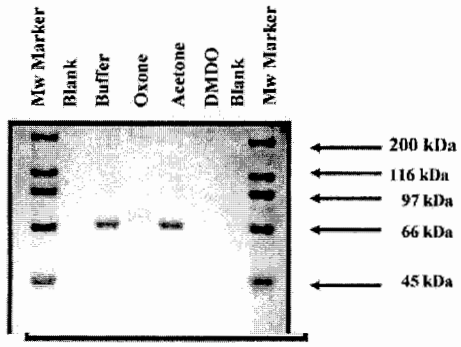

FIGURE 5. Comparison of degradation of BSA with 10% buffered Oxone and DMDO solutions after a 1 min exposure at 22 °C.

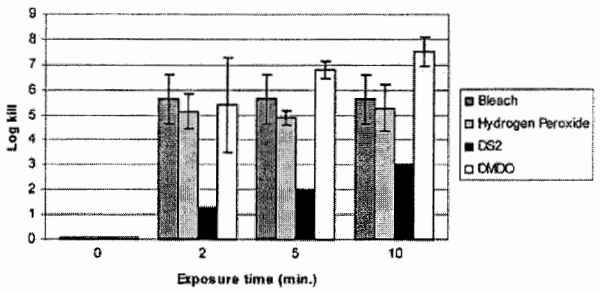

FIGURE 6. Comparison of inactivation of *B. atrophaeus* spores with a 10 min exposure to DMDO and conventional decontaminating solutions, 5.5% household bleach, 35% hydrogen peroxide, and neat DS2. Error bars indicate standard deviation of three replicates. DS2 experiments were performed once.

between susceptible amino acids. The mechanism of protein degradation by aqueous Oxone and DMDO is currently being investigated.

Comparison to Conventional Decontaminant Solutions. Sodium hypochlorite (bleach) and DS2 are decontaminant solutions used by the military. Aqueous solutions of hydrogen peroxide are commonly used for disinfection and sterilization. The effectiveness of DMDO against spores of *B. atrophaeus* was compared to the effectiveness of these conventional decontaminant solutions (Figure 6). Results indicated that DMDO seems to have a comparable, if not greater, capacity for spore inactivation and achieved a higher level of inactivation at a 10 min exposure time in comparison to equal volumes of the other decontaminant solutions. Therefore, DMDO compared very favorably with the other decontaminant solutions in terms of effectiveness and time while potentially avoiding corrosive action.

Investigation of Stability of DMDO Solutions. It is imperative to assess the long-term effectiveness of the DMDO solution once it is produced to establish a protocol for its application and design the corresponding equipment. To assess this stability, Oxone and DMDO solutions were prepared in an open system and allowed to age for predetermined lengths of time before exposure to *B. atrophaeus* spores.

The effectiveness of the DMDO solutions decreased continuously with time, but a significant capacity for neutralization was still present after an hour of aging (Figure 7). This loss of activity can be attributed to at least two major causes: volatilization and chemical decomposition. Experiments in this laboratory have established the presence of a significant partial pressure of gaseous DMDO above the generating solution, indicating the volatile nature of DMDO (*14*). The decomposition of DMDO is a second-order reaction that is very sensitive to pH, ionic strength, and the presence of transition metal impurities (*29*).

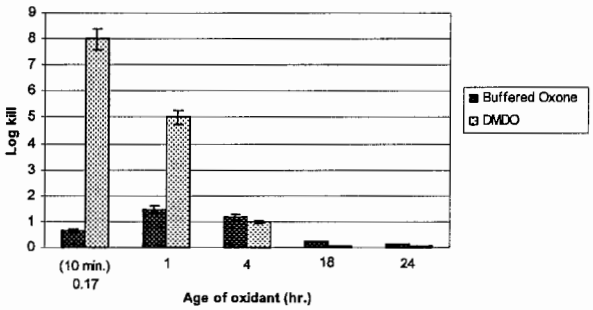

FIGURE 7. Inactivation of *B. atrophaeus* spores with a 10 min exposure to aged 10% buffered Oxone and DMDO solutions. Error bars indicate standard deviation of three replicates.

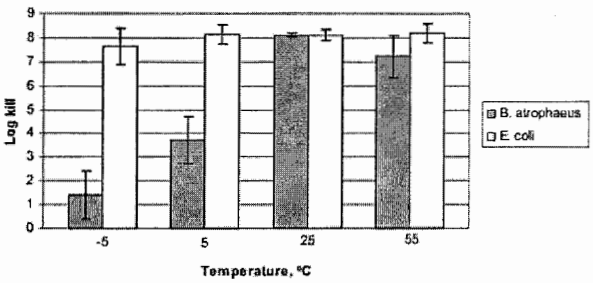

FIGURE 8. Inactivation of *B. atrophaeus* spores and *E. coli* cells with a 10 min exposure to DMDO solutions at different temperatures. Error bars indicate standard deviation of three replicates.

These results dictate that solutions of DMDO should be used shortly after preparation, preferably within an hour. This would favor keeping the chemical components separated prior to combination with water, thus ensuring maximum effectiveness and minimum logistic requirements (volume and weight). If necessary, the appropriate amount of acetone could be premixed with the appropriate amount of water to minimize flammability.

Effect of Temperature on DMDO Solutions. A decontaminant solution must be effective over the range of ambient temperatures likely to be encountered during normal use. The effectiveness of DMDO against *E. coli* cells shows little variation over the range of temperature studied (−5 to 55 °C) (Figure 8). However, a decrease in effectiveness is observed during inactivation of bacterial spores at both high and low temperatures. At −5 °C, the targeted inactivation rate was achieved by increasing both the reaction time of acetone with the Oxone solution before application and the contact time of the spores with the DMDO solution. The observed effect can be overcome by optimizing DMDO solution temperature and/or reaction times with respect to field conditions.

The potential decrease of spore inactivation at the highest temperature may be attributed to the volatility of both acetone and DMDO. Repeated applications of the decontamination solution to the targeted surface would alleviate this issue by maintaining an effective level of applied active chemical and by surface cooling.

Treatment of Tire Surfaces. An effective decontamination solution must be able to penetrate contaminated material and maintain its biocidal effectiveness while in contact with the substrate. Vehicle tire material was selected as a representative substrate because of its potential to widely disseminate biological agents. The shape of the treads and the potential reaction of the rubber with DMDO are important factors to consider during application of decontaminant solutions.

A 10 min DMDO treatment of tire coupons contaminated with bacterial spores resulted in greater than 6-log kill for

spores of *B. atrophaeus* and in 7-log kill for the spores of *B. thuringiensis*. A darkening of the solution was observed after prolonged soaking of tire coupons in the DMDO solution for as long as 12 h. This was likely due to extraction of rubber components. There was no apparent physical damage to the tire coupon, which retained its original flexibility. This indicated that DMDO reacted faster with the spores than with the rubber tire surface, retaining its full decontamination potential while not altering the physical characteristics of the tire. These experiments, although preliminary, suggest that DMDO solutions will have minimal adverse impact on vulcanized rubber surfaces, particularly given the short exposure time required for effective decontamination.

DMDO is a highly effective biological decontaminant and achieves rapid kill rates for viruses, bacteria, fungal spores, and bacterial spores. Solutions of DMDO offer a superior alternative over existing decontaminants because of its effectiveness at neutral pH conditions and its lack of toxic byproducts.

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14. ABSTRACT

Dimethyldioxirane (DMDO), generated in situ by adding acetone to an aqueous solution containing potassium peroxymonosulfate (Oxone) at neutral pH, was investigated for inactivation of biological warfare agent simulants. The DMDO solution inactivated bacterial spores, fungal spores, vegetative bacterial cells, viruses, and protein by 7 orders of magnitude in less than 10 min. The kill rates of DMDO were more pronounced when compared to kill rates of buffered Oxone alone. Conditions for the use of DMDO as a biological decontaminant were optimized by evaluating the effects of age and temperature on open systems. DMDO effectiveness was compared to that of current decontaminant solutions such as DS2 (used by the U.S. military), bleach and hydrogen peroxide and was shown to be superior in achieving a 7-log kill of Bacillus atrophaeus, a Bacillus anthracis spore simulant. The results demonstrate the potential for DMDO to fill the need for a noncorrosive, nontoxic and environmentally safe decontaminant.

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